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Immune response and protection provided by live tachyzoites and native antigens from the NC-6 Argentina strain of *Neospora caninum* in pregnant heifers

Y.P. Hecker[a], D.P. Moore[a], V. Quattrocchi[b], J. Regidor-Cerrillo[c], A. Verna[d], M.R. Leunda[d], E. Morrell[d], L.M. Ortega-Mora[c], P. Zamorano[b], M.C. Venturini[e], C.M. Campero[d,*]

[a] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*
[b] *Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, CC 77, 1708 Morón, Argentina*
[c] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*
[d] *Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, CC 276, 7620 Balcarce, Argentina*
[e] *Facultad de Ciencias Veterinarias de la Universidad Nacional de La Plata (UNLP), 1900 La Plata, Argentina*

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ABSTRACT

The aim of the present study was to compare the immunogenicity and protective efficacy of live tachyzoites and native antigen extract obtained from the NC-6 Argentina strain against vertical transmission of *Neospora caninum*, following experimental challenge in pregnant heifers with the NC-1 strain. Sixteen pregnant heifers were divided in 4 groups of 4 animals, each receiving different inoculation before mating: group A animals were intravenously (iv) inoculated with 6.25×10^7 live tachyzoites of the NC-6 strain, group B heifers were inoculated twice subcutaneously (sc) with *N. caninum* native antigen extract formulated with ISCOMs, group C heifers were sc injected with sterile phosphate-buffered saline (PBS) and group D heifers received sc ISCOM-matrix (ISCOMs without antigen). All groups were iv challenged with the NC-1 strain at 70 days of gestation. Serum and heparinized blood samples were collected eight times on weeks 0, 2, 3, 5, 9, 13, 16 and 17 post-inoculation. Dams were slaughtered at the 17th week of experiment (104 days of pregnancy) and placental and fetal tissue samples were collected. Specific antibody responses in heifers were tested by indirect enzyme linked immunosorbent assay (iELISA). The cellular immune response in dams was assessed by quantifying IFN-γ production and the percentages of T-cells ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) and monocytes in peripheral blood mononuclear cells (PBMC). Fetal fluids and tissue samples were tested using the indirect fluorescence antibody test, western blot, histopathology, immunohistochemistry and nested-PCR. A significant increase in *N. caninum* antibody response was detected in heifers of groups A and B from week 3 after inoculation ($P < 0.001$). IFN-γ production was similar in groups A and B at week 13 ($P > 0.05$). All fetuses were viable at necropsy. Specific IgG against *N. caninum* was detected in 1/4 fetal fluids recovered from groups A, C and D heifers and 3/4 fetal fluids from group B. Transplacental transmission could be determined in one fetus from group A and three fetuses from group B by nPCR. All fetuses from groups C and D were positive by nPCR. It is noteworthy

* Corresponding author. Tel.: +54 2266 439100; fax: +54 2266 439101.
E-mail address: ccampero@balcarce.inta.gov.ar (C.M. Campero).

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that dams with higher $CD4^+/CD8^+$ ratios in PBMC, regardless of the experimental group, had lower pathology scores. The results of this study confirm that inoculation with live parasites pre-mating may provide at least partial protection against vertical transmission of *N. caninum* following challenge in heifers at early gestation.

1. Introduction

Neosporosis is a disease caused by the obligate intracellular protozoan *Neospora caninum* that results in abortions in cattle (Dubey and Schares, 2011). Transplacental or vertical transmission of the parasite from an infected dam to its fetus is the major natural route of infection (Dubey et al., 2007). More than one billion dollars per annum are lost due to bovine neosporosis in the cattle industry worldwide (Reichel et al., 2013). The control strategy for this disease is based on general biosecurity procedures, in order to interrupt the cycle of the parasite, and the culling of the seropositive cattle if economically viable (Reichel and Ellis, 2002). Indeed, the development of other control measures like treatment or vaccines is urgently needed.

Host resistance to *N. caninum* is associated with a T helper 1 immune response mediated by cytotoxic T lymphocytes and the production of interferon-gamma (IFN-γ), interleukin-12 (IL-12), tumor necrosis factor (TNF) and immunoglobulin G_2 (IgG_2) (Staska et al., 2003; Innes, 2007). The reduction of vertical transmission and abortions in subsequent pregnancies suggests the existence of protective immune mechanisms (Dubey et al., 2007). A vaccine that mimics the immune response generated naturally (Moore et al., 2005) or by inoculation with live parasites (Innes et al., 2001; Williams et al., 2007) may prevent *Neospora*-associated abortion. McAllister et al. (2000) showed clearly that naturally exposed cattle developed protective immune mechanisms against abortions in a subsequent *Neospora*-related outbreak.

The progress achieved with live vaccines against other protozoa like *Toxoplasma gondii*, *Babesia* spp. and *Coccidia* spp. (Buxton, 1993; Bock et al., 2004; Shirley et al., 2005) encourages further research to develop a live vaccine to prevent bovine neosporosis. Live vaccines have disadvantages such as the risk of reversion to virulence and the lack of stability for long term storage (Reichel and Ellis, 2009; Weber et al., 2013). Many studies evaluating inactivated vaccines have been largely unsuccessful in protecting cattle against experimental challenge (Andrianarivo et al., 1999, 2000; Williams et al., 2007) or in cattle that are naturally persistently infected (Andrianarivo et al., 2005). Epidemiological data suggest that a low efficacy (<50%) could be achieved using a killed vaccine under field conditions but this varied from farm to farm (Romero et al., 2004; Weston et al., 2012). It is noteworthy that in one of those farms where frequent abortions due to *N. caninum* were recorded, the killed vaccine had an efficiency of 61% (Weston et al., 2012).

Many adjuvants have been evaluated in the formulation of a *Neospora*-inactivated immunogen to be used in cattle (Andrianarivo et al., 1999; Choromansky and Block, 2000; Moore et al., 2005; Williams et al., 2007). Immune stimulating complexes (ISCOMs) showed promising results when IFN-γ levels were compared between live and inactivated *Neospora* immunogens in calves (Moore et al., 2011). Interestingly, partial protection against tissue cyst formation was provided by using crude rhoptries antigens from *T. gondii* formulated in ISCOMs in pigs (García et al., 2005).

Previous studies have shown that immunization using NC-Nowra, an Australian attenuated strain of *Neospora* isolated from a congenitally infected calf (Miller et al., 2002), can protect against transplacental transmission and abortion following an experimental challenge with a virulent strain of *N. caninum* during pregnancy in cattle (Williams et al., 2007; Weber et al., 2013).

The NC-6 strain of *N. caninum* is an Argentinean isolate that was obtained from the feces of a naturally infected dog (Basso et al., 2001). The ability of the NC-6 strain to induce protection in pregnant challenged heifers has not been yet evaluated. The aim of the current work was to describe the immune responses and efficacy against vertical transmission generated after vaccination with live tachyzoites or native antigens obtained from the NC-6 Argentina strain following experimental challenge in pregnant heifers with NC-1 at day 70 of gestation.

2. Materials and methods

2.1. Animals and experimental design

Thirty-one seronegative 22 month old Angus heifers were initially involved in the experiment. The heifers belonged to a beef herd located at INTA Balcarce, Argentina. Heifers were bled monthly for a year prior to breeding and their seronegative status to *N. caninum* was confirmed using an indirect fluorescent antibody test (IFAT) (negative at 1:25) (Venturini et al., 1999). In addition, heifers were seronegative to *T. gondii* in a micro-agglutination test (Desmonts and Remington, 1980), and by serum neutralization tests for Bovine Viral Diarrhea and Infectious Bovine Rhinotracheitis. The herd was also brucellosis and tuberculosis free and a vaccination program against foot and mouth disease was routinely performed.

Heifers were in good body condition and maintained on pasture. Animals had adequate genital development and were cycling at the start of the trial. Routine clinical examination, pelvic area measurements and vaccination with inactivated vaccines against Bovine Viral Diarrhea Virus-1 (BVDV-1) and Bovine Herpesvirus-1 (BHV-1) (Bioabortigen, Biogénesis Bagó, Argentina) were performed twice two months before breeding.

Cattle were randomly allocated into four groups (Fig. 1). Group A heifers ($n = 9$) were immunized intravenously (iv) with 6.25×10^7 tachyzoites of NC-6 Argentina strain in sterile phosphate-buffered saline (PBS) (pH 7.2) 4 weeks prior to mating. Group B heifers ($n = 9$) were immunized sc

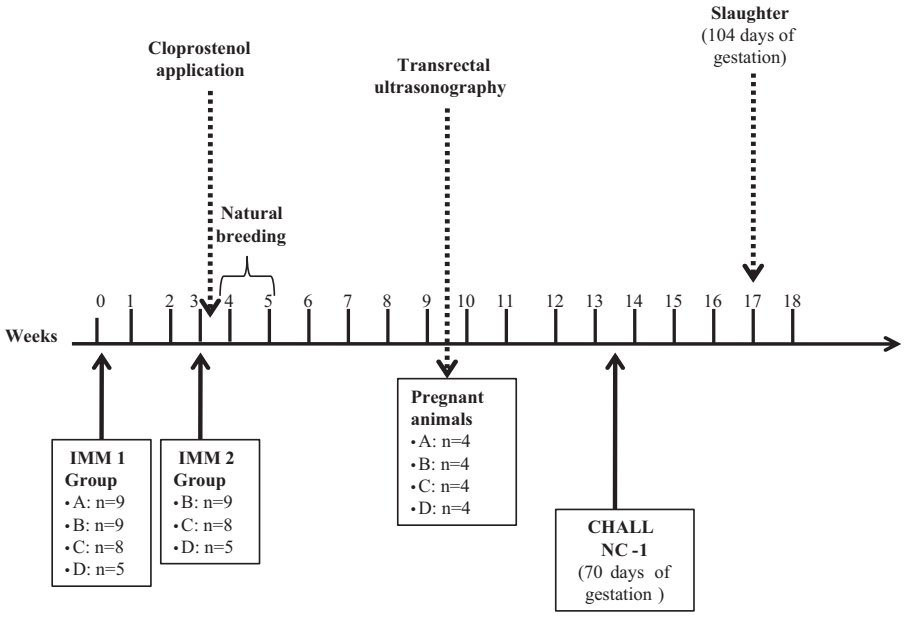


Fig. 1. Experimental design through time. This figure shows immunization (IMM1) with live tachyzoites (Group A) or experimental vaccine (Group B) (IMM1, IMM2) and challenge (CHALL) with a *N. caninum* NC-1 tachyzoites at 70 days of gestation.

in the neck region with 2 doses of a vaccine designed with a tachyzoite native antigen extract of NC-6 Argentina strain and ISCOMs (Abisco-300, ISCONOVA, Uppsala, Sweden) 3 weeks apart. Group C heifers ($n = 8$) were sham inoculated sc with sterile PBS and group D ($n = 5$) included heifers given sc ISCOMs alone following the same immunization regimen as group B. Groups A, B, C, and D were bred 4 weeks after the first immunization (Fig. 1). Local inflammatory reactions at the injection site were evaluated daily for one week after each inoculation.

Heifers were estrus synchronized using cloprostenol according to the manufacturer's instructions (D cloprostenol, Tecnofarm®, Argentina), then allocated into pens with four healthy Angus bulls (campylobacteriosis and trichomonosis-free) for natural breeding over 7 days. All the animals were fed on a natural pasture in one paddock and maintained under standard animal husbandry conditions. Clean water was available at all times. To confirm that cattle were pregnant, they were scanned by transrectal ultrasonography at 35 days after mating. From the 31 cattle, 16 pregnant animals, each carrying single fetuses, were selected for the experiment (Table 1).

All groups were challenged iv with 4.7×10^7 NC-1 tachyzoites at 70 days of gestation. Fetal viability was confirmed using ultrasonography to monitor fetal heartbeat and movement every week following challenge until slaughter at the 17th week of the experiment (104 days of pregnancy). All animals used in this study were handled in strict accordance with good animal practice and the conditions defined by the Animal Ethics Committee at INTA Balcarce. All efforts were made to minimize suffering during slaughter.

Table 1
Study design and treatment.

Groups	Pregnant animals challenged	Treatment
A	4	Live tachyzoites (6.25×10^7/ml of NC-6)
B	4	Native antigen extract of NC-6 (500 μg) + ISCOMs (750 μg)
C	4	PBS
D	4	ISCOMs

2.2. Samples of dams

Blood samples (40 ml), with and without heparin, were collected from the jugular vein for serological and cellular immune response assays at weeks 0, 2, 3, 5, 9, 13, 16 and 17 of the trial.

Blood samples with heparin were centrifuged 40 min to $4000 \times g$ to remove plasma; the layer of peripheral blood mononuclear cells (PBMC) was recovered. Cells were treated with ammonium chloride and centrifuged for 15 min at $2000 \times g$ (Corpa et al., 2001). Finally, PBMC were cryopreserved with 20% bovine fetal serum and 5% dimethyl sulfoxide (DMSO) and held at −80 °C until use. Blood samples without heparin were centrifuged at $1600 \times g$ for 10 min and serum was kept at −20 °C.

2.3. Culture and strain of parasite

Two different *N. caninum* isolates were used. Live tachyzoites of the NC-6 Argentina strain (Basso et al., 2001) were used to inoculate heifers of group A and for native antigen production for the vaccine in Group B. The NC-1 isolate of *Neospora* (Dubey et al., 1988) was used for challenge in all heifers. Both isolates were maintained under the same conditions by continuous passage on Vero cells and were harvested when 80% of cells were infected.

2.4. Parasite inocula

NC-6 tachyzoites were washed three times in PBS (pH 7.4), separated from host cell debris by sequential passage of the mixture through 21, 23, 25 and 27 gauge needles and filtered through Sephadex columns (Sephadex™ G-25 Medium, GE Healthcare, Sweden). Tachyzoite number was determined by counting two aliquots of the resulting filtrate using a hemocytometer. Then, animals from group A were inoculated with live tachyzoites adjusted to 6.25×10^7 tachyzoites of NC-6 in a 2 ml dose per animal. Parasite inocula were transported in an insulated box at room temperature (RT) for animal inoculation within 45 min after harvest from the tissue culture.

For challenge, NC-1 tachyzoites were harvested and viability was determined by counting in a hemocytometer. Organism numbers were adjusted to a concentration of 4.7×10^7 in a final volume of 3 ml of PBS, as previously described for NC-6, and used immediately to infect animals.

2.5. Native antigen extract

Native antigen extract used for the formulation of the experimental vaccine, serological assays and stimulation of whole blood for assessment of IFN-γ production was obtained as previously described by Innes et al. (1995). Briefly, 1×10^9 NC-6 tachyzoites were purified using Sephadex columns (GE Healthcare) and pelleted by centrifugation at $1500 \times g$ for 10 min. All parasite pellets were re-suspended in 1 ml of 10 mM Tris hydrochloride pH 7.0 containing 2 mM of phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA), disrupted by three freeze–thaw cycles, and sonicated with 6× 30 s bursts on ice at maximum setting using a sonic cell disruptor (Sonifier 450, Branson Ultrasonic Co., USA). Protein content was determined using the Micro BCA protein assay method (Pierce, Rockford, USA) and the supernatant aliquoted and cryopreserved at −80 °C.

2.6. Native antigen extract of tachyzoites into ISCOMs

Each vaccine dose was formulated as a mixture containing 250 μl of native antigen extract (estimated to contain 500 μg/ml) and 200 μl of ISCOMs (with approximately 750 μg/ml), kindly provided by Dr. Morein (ISCONOVA). Formulations were maintained in an insulated box at RT for one hour until vaccination.

2.7. Indirect ELISA for detecting specific IgG and its subisotypes

N. caninum-specific IgG and isotypes IgG_1 and IgG_2 were determined by iELISA (Moore et al., 2011). Briefly, 96-well plates were coated with native antigen extract (1 μg in 100 μl/well) and bovine serum samples were diluted (1:100) in PBS/0.75 M EDTA/EGTA (pH 6.3) plus 4% skimmed milk. Anti-bovine IgG polyclonal antibody conjugated to peroxidase (diluted 1:1000; Sigma Chemical Co.) was used as a secondary detection antibody. For each plate, a negative (IFAT titer < 1:25), a mid-positive (IFAT titer = 1:100), and a high-positive (IFAT titer > 1:200) control were included. Total bovine IgG was evaluated using a kinetic reading (Multiskan RC, Labsystems, Helsinki, Finland) at an optical density of 405 nm (OD 405), when the *N. caninum* high-positive control reached 1.0 ± 25%. Serum samples were analyzed in duplicate and the mean value of the optical density (OD) was converted into a relative index percent (RIPC) using the following formula: RIPC = (OD405 sample − OD405 negative control)/(OD405 positive control − OD405 negative control) × 100. A RIPC value ≥ 8.2 indicated a positive result (Rojo-Montejo et al., 2009).

In addition, anti-bovine IgG_1 or IgG_2 mAbs (1:100; Serotec™, Oxford, UK) were used as described (Moore et al., 2011). For IgG_1 and IgG_2, a kinetic reading was determined at an OD 405 when the *N. caninum* high-positive control with anti-IgG_1 reached 1.0 ± 25%. Data were expressed as a ratio of OD values for IgG_1OD/IgG_2OD.

2.8. IFN-γ assay

Immune stimulation was performed as previously described (Serrano-Martínez et al., 2007). Briefly, duplicate aliquots of 0.9 ml of heparinized blood, collected from each heifer at weeks 0, 2, 3, 5, 9, 13, 16 and 17, were cultured in duplicate wells in 24-well tissue culture plates (Cellstar Greiner, Monroe, USA). Cells were also cultured with 0.1 ml of PBS (unstimulated control) or concanavalin A (Con-A, Sigma) at 10 μg/ml to assess their ability to respond to stimulation and secrete IFN-γ, or with native antigen extract from the NC-6 strain (10 μg/ml). Plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Culture supernatants were collected and assessed for IFN-γ content using a commercial sandwich iELISA (BOVIGAM, CSL, Victoria, Australia) following manufacturer's instructions. IFN-γ concentrations in supernatants were analyzed using a spectrophotometer (Labsystems Multiskan Plus). A standard curve, derived from a series of dilutions of a recombinant bovine standard (Serotec™, Oxford, UK), was used to estimate IFN-γ levels in the test samples.

2.9. Phenotypic analysis of PBMC using flow cytometry

Percentages of T-cells ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) and monocytes in PBMC were analyzed using flow cytometry after immune-labeling with mAbs specific for bovine leukocyte subpopulations: CC8 (IgG_{2a}, anti-CD4), CC63 (IgG_{2a}, anti-CD8), CC-G33 (IgG_1, anti-CD14), CC15 (IgG_{2a}, anti-$\gamma\delta^+$) (AbD Serotec) and secondary antibodies rat anti-bovine IgG isotypes conjugated with FITC or PE (AbD Serotec).

Cell suspensions (5×10^5 cells/well) were incubated with mAbs diluted in PBS at 4 °C for 20 min. Cells were then washed with PBS, re-suspended in 100 μl of fluorescence-activated cell sorting (FACS) buffer flow (Becton, Dickinson and Company, Franklin Lakes, USA) and fixed with 0.05% formaldehyde. Isotype controls were used as negative controls. Analysis was performed using a FAC Scan cytometer and Cell Quest software (Becton, Dickinson and Company). Ten thousand cells were analyzed and the results expressed as the percentage of positively immunolabeled cells.

2.10. Necropsy, sampling, histopathology and immunohistochemistry

Following slaughter fetal and placental tissues were immediately removed and examined following standard gross pathology procedures (Campero et al., 2003). Fetal length was estimated using crown-rump measurement (Kirkbride, 1986).

Fetal tissue samples including lung, spleen and abomasal content were extracted to determine presence of aerobic and microaerophilic organisms, BVDV and BHV, and *Campylobacter fetus* and *Tritrichomonas fetus*, according to methods described by Campero et al. (2003).

Fetal cavity fluids were collected and stored at −20 °C until assessment by IFAT (Caspe et al., 2012).

Placental and fetal tissue samples were collected for DNA extraction and histopathological analysis. Fetal tissues, including whole CNS, liver, heart, lung, striated muscle, periorbital muscle, adrenal gland, kidney, small and large intestine, and spleen, were collected and fixed in 10% buffered formalin, processed by standard methods and embedded in paraffin blocks. Five μm-thick sections of tissue were cut, mounted on glass microscope slides and stained with hematoxylin and eosin (H&E). Similarly, placental samples were taken from different placentomes and the inter-cotyledonary chorion (5 samples of every region). Maternal CNS samples were also taken and analyzed.

Based on the severity of lesions, four scores for histological lesions were established: absent (0), mild (1), moderate (2) and severe (3) according to Caspe et al. (2012). Based on the severity of the lesions, a pathology score was calculated by adding individual lesion scores assessed in the CNS, heart, lung, liver and placenta (minimum score = 0; maximum score = 15). Finally a mean fetal pathology score from each group was calculated.

Tissues where *N. caninum*-compatible lesions were observed were selected and analyzed by immunohistochemistry (IHC) using a polyclonal antibody raised against NC-1 tachyzoites and an avidin–biotin complex procedure (ABC) (Vectastain Elite ABC kit; Vector Laboratories, USA) as previously described (Campero et al., 2003).

2.11. IFAT on fetal fluids

IFAT was performed using a fluorescein isothiocyanate labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma) (Dubey et al., 1988). *N. caninum* specific antibodies were measured in fetal fluids using a dilution of 1:10 to endpoint titers (Caspe et al., 2012). Positive and negative control sera were used. Slides were examined with an epifluorescence microscope (Olympus Bx 51, Olympus Inc., Tokyo, Japan). Antibody titers were expressed as the reciprocal of the highest serum dilution that showed complete peripheral fluorescence of tachyzoites (Paré et al., 1995).

2.12. Immunoblotting analysis of fetal fluids

N. caninum-specific antibodies were detected in fetal fluids as described by Álvarez García et al. (2002). Briefly, nitrocellulose membranes (Immobilon-NC, 0.45 μm, Millipore, USA) with *N. caninum*-native antigen were incubated with fetal fluids diluted to 1:50 and then incubated with an anti-bovine IgG HRP-conjugated rabbit antibody (Sigma Chemical Co.) at a dilution of 1:2000. Following incubation, membranes were washed and antigen–antibody reaction was visualized using 4-chloro-1-naphthol (Sigma Chemical Co.).

2.13. IFN-γ production in fetal fluids

IFN-γ production was measured in peritoneal fluids collected from fetuses using the IFN-γ kit (BOVIGAM). A standard curve, derived from a series of dilutions of a recombinant bovine IFN-γ standard (Serotec™, Oxford, UK) was used to estimate IFN-γ levels in the test samples. The mean OD values were plotted against the units/ml of recombinant IFN-γ. A regression line was calculated and the quantity of IFN-γ present in each sample was determined from the standard curve.

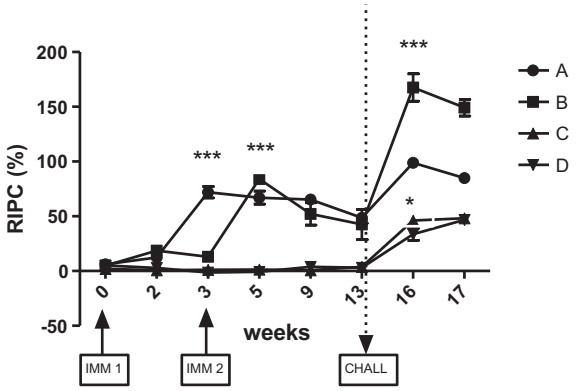
2.14. Nested PCR (nPCR)

DNA was isolated from tissue samples using a commercially available kit according to the manufacturer's recommendations (DNeasy Tissue Kit, QIAGEN Group, Germany). DNA concentration was measured using an Epoch micro-volume spectrophotometer system (Epoc, Bioteck® Instruments, Inc., Vermont, USA) and samples were diluted to a final concentration of 60 ng/μl for DNA detection by nPCR. For detection of parasite DNA, an nPCR on the internal transcribed spacer (ITS1) region of *N. caninum* was carried out with four oligonucleotides as described by Buxton et al. (1998). Secondary amplification products were visualized by electrophoresis in 2% agarose gel and ethidium bromide staining. DNA equivalent to 10^2 tachyzoites was used as the positive PCR control. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms employing different sets of instruments, aerosol barrier tips and disposable gloves. Moreover, negative control samples were included in each set of DNA extractions and PCR reactions.

2.15. Statistical analysis

RIPC values from serum antibody responses and IFN-γ production were compared between groups by using PROC-MIXED SAS for one-way repeated measures analysis of variance (ANOVA) with treatment as the grouping factor and time as the repeated measures factor (Littell et al., 1998).

Parasite detection frequency was compared by Fisher's exact test. Lesion score values were analyzed using the Kruskal–Wallis test followed by a non-parametric multiple-comparison test. A regression line was calculated between the ratio of $CD4^+/CD8^+$ cells from each dam and the pathology score of its fetus. All statistical analyses were performed using Graph-Pad Prism 5 v.5.01 (GraphPad Software, San Diego, CA, USA).



* $P < 0.05$ *** $P < 0.001$

Fig. 2. Antibody responses presented as a mean of the values, mean relative index percent (RIPC) and error bars, in cattle after immunization (IMM1, IMM2) with live tachyzoites (Group A) or experimental vaccine (Group B) and challenged (CHALL) with *N. caninum* NC-1 tachyzoites at 70 days of gestation. Positive cut-off ≥ 8.2 RIPC. *$P < 0.05$; ***$P < 0.001$.

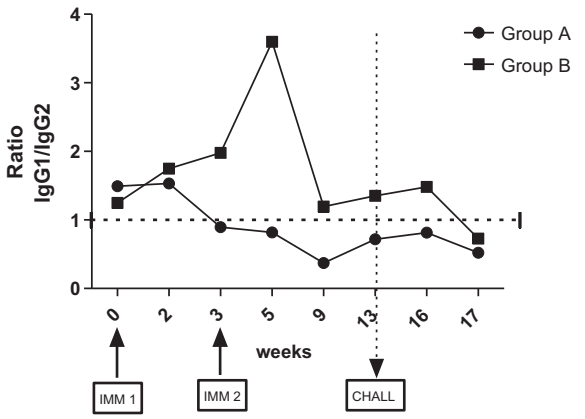


Fig. 3. Ratio of *Neospora*-specific IgG_1/IgG_2 antibodies after immunization (IMM1, IMM2) of animals in Group A (inoculated with live tachyzoites) and in Group B (inoculated with *Neospora*-native antigen extract formulated with ISCOMs) and challenged (CHALL) with *N. caninum* NC-1 tachyzoites at 70 days of gestation. Control groups are not shown.

3. Results

No apparent clinical signs were observed in any heifer throughout the study. No significant local reactions were observed at the site of injection in heifers receiving the experimental vaccine.

3.1. Serum antibody responses

3.1.1. Specific total IgG

RIPC values obtained by iELISA are shown in Fig. 2. *Neospora*-specific antibodies were detected in both group A and B heifers beginning at week 3 after immunization although RIPC values were significantly higher in group A when compared with group B heifers ($P < 0.001$). From week 5 until week 13, no significant differences were observed between these two groups ($P > 0.05$); however, their RIPC values were different than those determined in heifers from groups C and D ($P < 0.001$) (Fig. 2). After NC-1 challenge, RIPC values increased significantly in all animals from all groups ($P < 0.001$). The animals from group B had the highest antibody titers post challenge and were significantly different from all others ($P < 0.001$). At week 17, RIPC values of animals from groups D and E also increased but were lower than group A and B heifers ($P < 0.05$).

3.1.2. IgG subisotypes

Ratios of *N. caninum* specific IgG_1 and IgG_2 antibody responses are shown in Fig. 3. Group A heifers showed ratios close to or <1; in contrast, ratios >1 were observed in heifers from group B until week 16. By week 17 (4 weeks post challenge) heifers from all experimental groups had ratios <1.

3.2. IFN-γ production in whole blood of heifers

There was neither specific IFN-γ production nor differences in the responses among the experimental groups at week 0 ($P > 0.05$). However, an increased IFN-γ production at similar levels was detected in heifers from groups A and B at week 13 ($P > 0.05$). These IFN-γ responses in dams from groups A and B were significantly different compared with group D. IFN-γ production in whole blood stimulated with PBS was lower than that observed in whole blood stimulated with *N. caninum* lysate at all time points throughout the experiment. However, no significant differences between experimental groups ($P > 0.05$) were determined in the whole blood stimulated with *N. caninum* lysate.

3.3. Phenotypic analysis of PBMC using flow cytometry

The most notable change in PBMC subpopulations was a decrease in percentages of $CD4^+$ cells in heifers from groups A, B and D when compared with group C. However, this decrease was not significant until week 13 ($P > 0.05$). These changes were more evident after the NC-6 inoculation in heifers from group A (week 3) and after the second dose of the experimental vaccine (week 5) in heifers from group B. At week 5, PBMC collected from group A and group B had lower percentages of $CD4^+$ cells compared with heifers from groups C and D, but differences among these groups were not statistically significant at any time (data not shown). Percentages of $CD8^+$ cells were lower throughout the experiment in heifers from groups A and B compared with groups C and D, although there were no statistically significant differences between groups.

$CD4^+/CD8^+$ percentage ratios were analyzed throughout the experiment (Fig. 4). In group B the $CD4^+/CD8^+$ ratios were lower compared with those observed in group A heifers. After inoculation either with live parasite or native antigen extract plus ISCOMs, $CD4^+/CD8^+$ ratios decreased until time of NC-1 challenge (week 13) where the ratios increased slightly and later decreased again.

The percentages of γδ+ T cells and monocytes did not change between treatments or over time ($P > 0.05$).

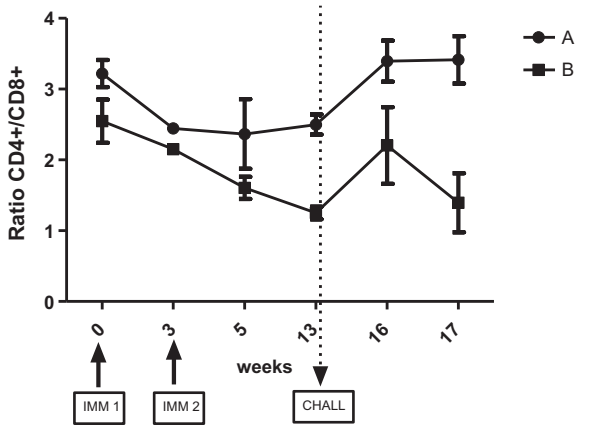


Fig. 4. Ratio of CD4+/CD8+ T cells in cattle after immunization (IMM1, IMM2) with live tachyzoites (Group A) or experimental vaccine (Group B) and challenge (CHALL) with *N. caninum* NC-1 tachyzoites at 70 days of gestation. Control groups are not shown.

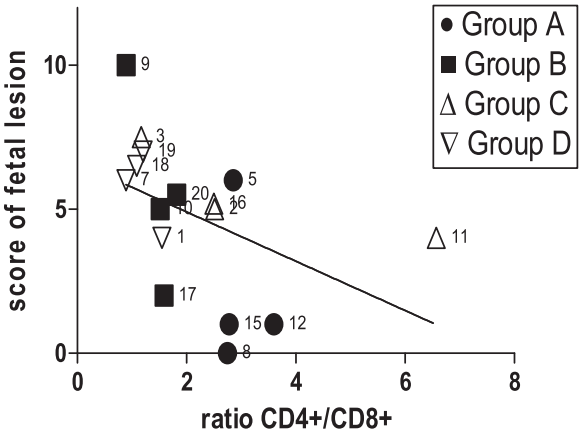


Fig. 5. Mean CD4+/CD8+ ratio versus mean fetal lesion score in week 16. The numbers correspond to fetuses of different groups: A (#5, #8, #12, #15), B (#9, #10, #17, #20), C (#2, #3, #11, #16), D (#1, #7, #18, #19).

3.4. Fetal viability

All fetuses were viable and no gross lesions were observed following slaughter and *post mortem* examination.

3.5. Fetal and placental histopathology

N. caninum characteristic lesions were scored according to severity (Table 2). Mild lesions (grade 1) were observed in 3 out of 4 fetuses recovered from group A heifers in heart and placenta (fetus #15) and lung and placenta (fetus #12). Only one fetus (fetus #5) showed multifocal necro-hemorrhagic hepatitis and mononuclear pericarditis (grade 2) and mild lesions (grade 1) in CNS, lung and placenta. On the other hand, fetuses from groups B, C and D showed lesions ranged from absent to severe (Table 2). The lowest mean pathology score was assessed in group A (mean = 2.75; standard error = ±2.99) when compared with groups B (7.5; ±4.5), C (7.75; ±1.5) and D (7.5; ±1.29) ($P < 0.05$). In addition, group A had a lower pathology score and a higher ratio CD4+/CD8+ than groups B, C and D (Fig. 5).

No *N. caninum* antigen was detected using IHC on fetal tissues from group A. Positive labeling was observed in one fetus from group B in CNS samples (#9) and another from group D in lung tissue (#1). In addition, positive *N. caninum* labeling was detected in the placenta from two dams from group C and one from group D.

3.6. Specific N. caninum antibodies in fetal fluids

Specific *Neospora* antibodies were detected by IFAT in peritoneal fluid collected from 1 out of 4 fetuses from groups A, C and D, and in 2 out of 4 fetuses from group B.

A 17–18 kDa antigen was detected by western blot in all the IFAT positive fetuses. Two IFAT negative fetuses (one of group B and one of group D) showed the same band. Immunoreactivity with *N. caninum* antigens was not detected in fetal fluids from fetuses of group A.

3.7. IFN-γ in fetal fluids

No IFN-γ was detected in peritoneal fluid sampled from group A fetuses. IFN-γ was found in peritoneal fluid of 2 out of 4, 4 out of 4, and 3 out of 4 fetuses from groups B, C and D, respectively.

3.8. Nested PCR (nPCR)

Fetal CNS and lung, as well as placental caruncles, were samples where *N. caninum* DNA was detected more frequently using nPCR. *N. caninum* DNA was amplified from tissues collected from one fetus from group A, three fetuses from group B and all the fetuses from groups C and D.

There were significant differences in the frequency of detection of *Neospora* DNA from caruncles: group A (1/16), group B (8/16), group C (12/16) and group D (8/16) ($P = 0.0032$).

From each group, 16 fetal CNS samples were collected and *N. caninum* DNA was amplified in 1, 5, 3 and 6 samples from groups A, B, C and D, respectively, being significantly lower only in group A ($P < 0.001$). Similarly, when the fetal lung samples were analyzed, parasitic DNA was amplified in only 1 and 2 out of 16 samples from groups A and B, respectively, compared with 12 and 9 out of 16 in groups C and D, respectively. These results were significantly different when groups A and B were compared with groups C and D ($P < 0.0001$).

4. Discussion

Partial protection against vertical transmission in pregnant cattle challenged with NC-1 strain was provided only when live parasites of the NC-6 strain of *N. caninum* were inoculated before mating, as reported by others when performing the challenge with a heterologous strain (Weber et al., 2013). These results also suggest that decrease in protection against vertical transmission may occur if a heterologous challenge is carried out. Nevertheless, full protection against vertical transmission and abortion was reported when cattle was inoculated with

Table 2
Fetal results from all groups obtained by IFAT, WB, IFN-γ, PCR and histopathology of different tissues.

Groups	Treatment	Fetus #	Antibodies in fetal fluids[a]	IFN-γ in fetal fluids	nPCR	Pathology scores					Mean pathology score
						CNS	Liver	Heart	Lung	Placenta	
A	Live tachyzoites	5	+	–	+	1	2	2	1	1	2.75
		8	–	–	–	0	0	0	0	0	
		12	–	–	–	0	0	0	1	1	
		15	–	–	–	0	0	1	0	1	
B	Native antigen extract plus ISCOMs	9	+	–	+	2	2	3	3	3	7.5
		10	+	–	+	2	1	1	1	3	
		17	–	+	–	1	1	0	0	0	
		20	+	+	+	0	1	2	2	2	
C	PBS	2	–	+	+	0	0	2	3	2	7.75
		3	–	–	+	1	2	2	2	2	
		11	+	–	+	1	0	1	2	2	
		16	–	+	+	1	1	2	2	3	
D	ISCOMs	1	+	+	+	0	0	2	2	2	7.5
		7	+	+	+	0	1	2	3	2	
		18	–	+	+	1	2	2	1	1	
		19	–	+	+	2	1	2	2	2	

[a] Antibodies were detected by IFAT or Western blot.

NC-Nowra before mating and later challenged with NC-Liverpool (Williams et al., 2007).

In agreement with our previous work (Moore et al., 2011), this study showed that experimental vaccination with a native antigen extract from a lysate of *N. caninum* tachyzoites formulated with ISCOMs was immunogenic, but did not protect against vertical transmission after experimental iv challenge in pregnant heifers. Whether this new formulation protects against abortion remains to be evaluated. In an effort to examine all fetuses, their dams were sent to the slaughter before parturition. However, the inoculation of live tachyzoites using the iv route is an unnatural way to perform a challenge (Andrianarivo et al., 2000; Innes et al., 2001; Williams et al., 2007; Weber et al., 2013). Challenge using *N. caninum* oocysts via the oral route may be more appropriate to evaluate vaccine efficacy against exogenous transplacental transmission in cattle. Gondim et al. (2004) demonstrated that ingestion of *N. caninum* oocysts can cause transplacental transmission and abortion in cattle depending on the dose.

ISCOMs have been used successfully in the development of vaccines for ruminants (Morein et al., 2004). ISCOMs interact with dendritic cells and enhance cross-presentation of the incorporated antigens inducing efficient specific $CD4^+$ and $CD8^+$ T cell responses (Maraskovsky et al., 2009). Vaccines with ISCOMs as adjuvants are known to induce long-lasting antibody responses, a balanced Th1/Th2 response and induction of cytotoxic T lymphocytes (Lovgren Bengtsson et al., 2011). Pinitkiatisakul et al. (2005, 2007) demonstrated immunogenicity and protection against murine cerebral neosporosis after vaccination with recombinant NcSRS2 antigen incorporated into ISCOMs-Matrix. That formulation containing SRS2-ISCOMs induced Th1 and Th2 balanced responses. Similarly, Moore et al. (2011) reported that subcutaneous inoculation of native antigens of *N. caninum* formulated with ISCOMs induced higher titers of total IgG and similar levels of IFN-γ to those achieved after inoculation with live NC-1 tachyzoites. However, these immune responses were not sufficient to prevent vertical transmission (Andrianarivo et al., 2000; Williams et al., 2007). Although, a reduction of parasitemia and proliferation of *N. caninum* in the brains of immunized mice was associated with induction of specific antibodies against NcSRS2 formulated with ISCOMs (Pinitkiatisakul et al., 2005, 2007), the magnitude of systemic specific IgG response is also a good indicator of the magnitude of parasitemia (Innes et al., 2002) and the role of these antibodies in bovine neosporosis remains to be determined.

Different IgG subisotypes have been detected in pregnant cattle receiving inactivated vaccines (Andrianarivo et al., 1999, 2000) or in naturally infected cattle (Andrianarivo et al., 2005; Moore et al., 2005). Experimental infection of pregnant cattle with live parasites of *N. caninum* has been shown to induce a typical Th1 response, with antibody responses often biased toward the IgG_2 subisotype (Williams et al., 2000). In agreement with the previous report, cattle experimentally inoculated with live NC-6 in the present study (group A) had a predominant IgG_2 antibody response throughout the experiment. On the other hand, the serum IgG_1/IgG_2 ratio of heifers vaccinated using NC-6 extracts with ISCOMs (group B) was higher, indicating predominant levels of IgG_1 (except in week 17). Similar results were reported by Andrianarivo et al. (2000) and Moore et al. (2005), where high IgG_1 levels were detected in immunized heifers. In contrast, in mice high titers of both IgG_1 and IgG_2 were detected for all groups immunized with recombinant NcSRS2 in different ISCOMs formulations (Pinitkiatisakul et al., 2007). Altogether, these results prove the complexity of the immune mechanisms involved in the development of efficacious immunization against neosporosis. In addition to characterization of IgG subisotypes, the nature of T helper cell regulation should be analyzed in further studies.

In this work, animals from groups A and B showed similar IFN-γ levels, but a predominant IgG_2 response was

observed only in the first group. This lack of correlation between IFN-γ production and bovine IgG_2 subisotypes is in agreement with the results reported by Tuo et al. (2000) who suggested that production of bovine IgG_2 may be regulated by factors other than IFN-γ. In addition, Almería et al. (2009) described a predominant IgG_2 pattern not associated with IFN-γ production in cows that had aborted due to *N. caninum*. Rosbottom et al. (2007) reported increased percentages of circulating $CD4^+$ cells that expressed both IFN-γ and IL-4 showing that the clear Th1/Th2 dichotomy observed in several other species is rare in cattle.

In the present study, clear changes in lymphocyte populations were observed. Three weeks after inoculation in group A and two weeks after the second dose in group B, $CD4^+$ and $CD8^+$ T-cell populations decreased in PBMC in heifers compared with groups C and D (data not shown). However, in week 5 the $CD4^+/CD8^+$ ratio started to increase in group A and to decrease in group B, until NC-1 challenge when it increased again slightly. A transient state of T-cell hypo-responsiveness to *N. caninum* antigen and to mitogen has been previously reported in mice (Khan et al., 1997; Eperon et al., 1999) and cattle (Innes et al., 2001). In addition, Almería et al. (2003) observed a decline in lymphocyte subpopulations 2 weeks after experimental *Neospora* infection in heifers at day 110 of gestation. Although T-cell percentages decreased in PBMC, an increase in the percentages of T-cells was observed in internal organs from dams (Almería et al., 2003). In the present work, the T cell percentage was not determined in peripheral organs. Rosbottom et al. (2007) reported an increase in the percentages of circulating $CD4^+$ and $CD8^+$ T-cells after *Neospora* experimental infection. In that study, the inoculation of live NC-Liverpool tachyzoites was done in two groups at days 70 or 210 of gestation. In the current work, the inoculation of live NC-6 or vaccination with native antigen extract plus ISCOMs was performed prior to pregnancy. Nevertheless, over-interpretation of phenotypic analysis of PBMC, using flow cytometry, should be avoided because it is not an antigen-specific technique and we did not evaluate the variation of the T-cell populations in naïve pregnant cattle.

In this study all fetuses were viable at the time of slaughter, but presented different pathology scores. No vertical transmission was determined in 3 out of 4 fetuses of group A fetuses using IHC, IFAT and nPCR, suggesting protection in these dams that were inoculated pre-mating with live NC-6 tachyzoites. These findings are consistent with Williams et al. (2007) and Weber et al. (2013) who reported that calves born from mothers vaccinated with a live parasite were healthy and showed no evidence of congenital infection at birth. However, the remaining fetus of group A (#5) had moderate lesions, and was positive by IFAT and nPCR, showing that protection was not universally effective. Microsatellite characterization performed on 4 positive fetuses (one from each experimental group) allowed confirmation of infection by the NC-1 strain used in the challenge in 3 out of 4 fetuses (data not shown). Unfortunately, the low load of *Neospora*-DNA present in the infected fetus from group A did not allow genetic characterization of the strain. On the other hand, the moderate lesions observed in that specimen might not have been sufficient to affect fetal viability, suggesting that the fetus would have been born alive if the dam were not slaughtered.

The only fetal tissue from specimens in group B that had a similar frequency of parasite DNA compared to group A was the lung. Pescador et al. (2007) reported that distinct lesions were consistently observed in the lungs of aborted fetuses with positive IHC, suggesting a considerable degree of specificity for pulmonary tissue. In addition, in a BALB/C mouse model for experimental infection with *N. caninum*, parasite DNA was detected mainly in lungs during the early phase of infection (Rojo-Montejo et al., 2011), followed by a persistent stage with parasite presence in the brain (Collantes-Fernández et al., 2006).

Assessment of the ratio of $CD4^+/CD8^+$ cells versus fetal lesion score showed that those animals vaccinated with native antigen extract plus ISCOMs and those that received PBS and ISCOMs alone had more lesions and reduced ratio of $CD4^+/CD8^+$ than those animals receiving live NC-6 tachyzoites who had fewer lesions and a high ratio of $CD4^+/CD8^+$. According to the concept that $CD4^+$ cytotoxic T-cells and IFN-γ control *N. caninum* infection (Staska et al., 2003; Williams et al., 2007) it would be expected that animals that expressed a lower percentage of $CD4^+$ T cells should have more lesions in their fetuses. Further research needs to be performed at the materno-fetal interphase in order to expand on these findings.

In agreement with previous studies (Innes et al., 2001; Williams et al., 2007; Weber et al., 2013), this work showed clearly that the only way to prevent vertical transmission is by inoculating live parasites before mating. Unfortunately, the efficacy of both vaccine formulations to prevent the abortion was not evaluated in this study. Nevertheless, integrated herd management should probably include the use of inactivated vaccines to prevent abortions due to this parasite (Romero et al., 2004; Weston et al., 2012). The successful use of a non pathogenic strain of *T. gondii* to prevent ovine toxoplasmosis (Buxton, 1993) suggests that it should be possible to develop a vaccine to control bovine neosporosis.

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